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Rapid report

Analyses of a *Bacillus subtilis* homologue of the Na^+/H^+ antiporter gene which is important for pH homeostasis of alkaliphilic *Bacillus* sp. C-125

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Abstract

Bacillus subtilis was revealed to have a homologous region to the DNA fragment responsible for alkaliphily of alkaliphilic *Bacillus* sp. C-125 on the genome, as reported previously [1]. The *yufT* gene on the *B. subtilis* genome showed a significant similarity with ORF1 of *Bacillus* sp. C-125, which is related to membrane potential ($\Delta\Psi$)-driven Na^+/H^+ antiport activity and is important for pH homeostasis in an alkaline condition. Disruption of the *yufT* gene resulted in the decrease of Na^+/H^+ antiport activity, and the growth of the *yufT* disrupted strain was impaired with an increase in the external Na^+ concentration. We conclude that the *yufT* gene encodes a Na^+/H^+ antiporter, which has a dominant role in the extrusion of cytotoxic Na^+.

Keywords: Na^+/H^+ antiporter; pH homeostasis; Na^+ transporter; (*Bacillus subtilis*)

Sodium/proton antiporters are ubiquitous and have been proposed to play a wide variety of important physiological roles, including resistance to elevated levels of Na^+, pH homeostasis, osmoregulation, and signaling [2–4]. Extremely alkaliphilic *Bacillus* sp. strain C-125 requires membrane potential ($\Delta\Psi$)-driven Na^+/H^+ antiport activity encoded by ORF1 for pH homeostasis in an alkaline environment. The alkali-sensitive mutant 38154 of *Bacillus* sp. C-125, which contains a single ^{393}Gly to Arg substitution in the product of ORF1, lacks $\Delta\Psi$-driven Na^+/H^+ antiport activity and cannot retain a low internal pH under an alkaline condition [5,6]. Another alkali-sensitive mutant 18224 of *Bacillus* sp. C-125, which contains a ^{82}Gly to Glu substitution in the product of ORF3, still retains the ability to regulate of the internal pH, suggesting that ORF3 may not be involved in a Na^+/H^+ antiport system itself but be involved in a regulatory or associated function of the ion transport [7,8]. ORF1 and ORF3 are located close to each other within the same gene cluster on the genome of *Bacillus* sp. C-125 [6,8]. Recent genome sequence analyses found that *Bacillus subtilis* has a homologous region to the DNA fragment responsible for alkaliphily of *Bacillus* sp. C-125 including ORF1 and ORF3 [1]. In this report, to determine the possibility that a *B. subtilis* homologue of ORF1 (*yufT*) is responsible for Na^+/H^+ antiport activity and its role in neutralophilic *B. subtilis*, we disrupted the *yufT* locus and characterized the phenotype of the growth as a function of Na^+ concentration and pH, and the Na^+/H^+ antiport activity in right-side-out membrane vesicles of the disrupted mutant.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, *N*,*N*,*N*′,*N*′,-tetramethyl-*p*-phenylenediamine

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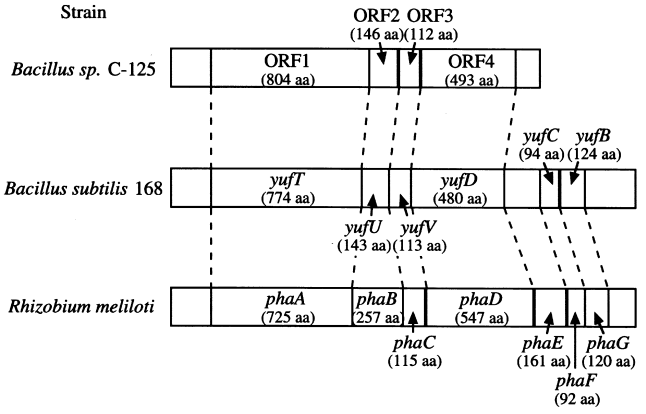

Fig. 1. Comparison of the organization of the *yuf* cluster of *B. subtilis* and two gene clusters involved in monovalent cation transport. The cluster of *Bacillus* sp. C-125 is involved in Na^+/H^+ antiport [6,8]. Tha *pha* gene cluster of *R. meliloti* is involved in K^+ transport [9]. Size of putative gene products is shown in parentheses.

Fig. 1 describes the organization of gene clusters involved in monovalent cation transport. *yufT*, *yufU*, *yufV*, and *yufD* on the *B. subtilis* genome correspond to ORF1, ORF2, ORF3, and ORF4, respectively, of *Bacillus* sp. C-125. They are very close in gene organization and protein size. The protein identity was 56.8% (ORF1 vs. YufT), 57.6% (ORF2 vs. YufU), 60.7% (ORF3 vs. YufV), and 51.5% (ORF4 vs. YufD). We also found homologies between the Yuf proteins and Pha proteins which have been recently shown to be involved in K^+ efflux in *Rhizobium meliloti* [9]: YufT, YufU, YufV, YufD, YufC, and YufB corresponded to PhaA, PhaB, PhaC, PhaD, PhaF, and PhaG, respectively. YufT and YufD are also related to NuoL and NuoM of *Escherichia coli* NADH:ubiquinone oxidoreductase (complex I), respectively [10]. From a high extent of hydrophobicity, YufT and YufD were predicted to be membrane proteins.

Bacillus subtilis 168 (*trpC2*) (kindly donated by M. Itaya) was used as the wild-type strain in this study. Disruption of the *yufT* locus was performed by double-crossover mutagenesis with the neomycin-resistant (Nm^R) gene. A 1.9 kb fragment containing a part of *yufT* was amplified by PCR with two specific oligonucleotide primers (yufTDR-F: GGGGGATCCATGGGCGCCATGCTCGG; yufTDR-R: GGCGTCGACATAACGAGAATGACATTGAC). The amplified DNA fragment was subcloned into the *Bam*HI and *Sal*I site of pUC19. The *Eco*RV site in the amplified fragment was converted into the *Xba*I site by linker ligation. The neomycin resistant gene (1.4 kb) excised from pBEST501 [11] was inserted into the converted *Xba*I site in *yufT*. The resultant plasmid was introduced into *B. subtilis* competent cells by transformation, and Nm^R single colonies were obtained. Southern hybridization and PCR analyses confirmed that the original *yufT* gene was replaced by the mutagenized *yufT* fragment and that insertion of the Nm^R gene did not occur at any other site. The *yufT* disrupted strain was named as SK001.

In most bacterial cells, the Na^+/H^+ antiporter is the major Na^+ extruding mechanism by the proton motive force (Δp). We measured Na^+ efflux from right-side-out membrane vesicles of the wild-type and SK001 upon energization by a transmembrane

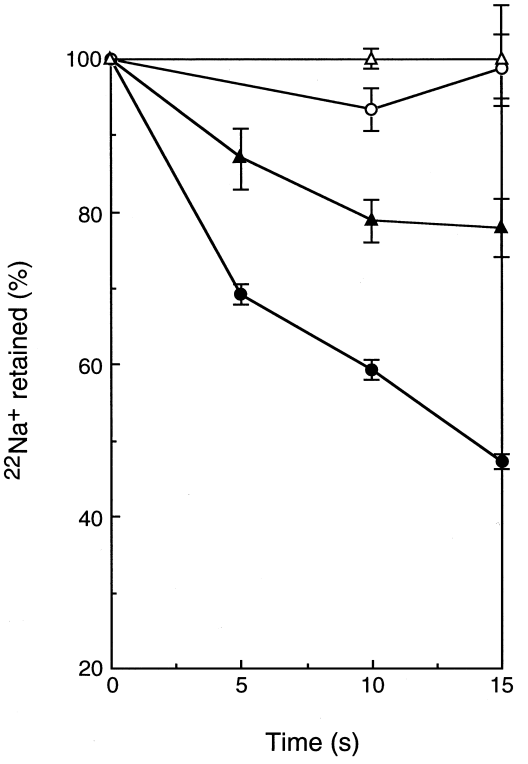

Fig. 2. Na^+ efflux from right-side-out membrane vesicles of wild-type and SK001 (*ΔyufT*) upon imposition of an inwardly directed proton gradient (ΔpH). Membrane vesicles were equilibrated with 100 mM potassium acetate containing $^{22}NaCl$ at pH 6. Na^+ efflux from vesicles of wild-type (circles) and SK001 (triangles) was initiated by dilution into buffers at pH 6 containing 100 mM potassium acetate (open symbols) or 100 mM potassium gluconate (closed symbols). Values are the average of five separate determinations; error bars indicate standard deviations.

proton gradient (ΔpH), which is one component of Δp (Fig. 2). ΔpH was created by imposing the outwardly directed acetate diffusion gradients [12]. The wild-type and SK001 were grown in LBK1/2 medium containing 10 g/l tryptone, 5 g/l yeast extract, and 5 g/l KCl at pH 7. Right-side-out membrane vesicles were prepared in 10 mM sodium phosphate buffer containing 100 mM potassium acetate and 5 mM $MgSO_4$ at pH 6 by following the osmotic shock procedure described by Kaback [13,14] and suspended in the same buffer at a final concentration of 10 mg of membrane protein per ml. Then, a small amount of carrier-free $^{22}NaCl$ (240 kBq) was added to 100 μl of the vesicle suspension, and the suspension was incubated on ice for 2 h in order to load $^{22}Na^+$ into the vesicles. $^{22}Na^+$ efflux determinations were initiated by diluting 2.5 μl of the membrane vesicle suspension into 2 ml of 10 mM potassium phosphate buffer containing 100 mM potassium gluconate and 5 mM $MgSO_4$ at pH 6, and quickly agitated. Under this condition, the theoretical 2.9 units of ΔpH (inside alkaline) were imposed across membrane vesicles. At given times, the reaction was terminated by filtering the diluted samples and the filter (0.45 μm pore size; Millipore Co.) was washed once with 4 ml of the dilution buffer. Radioactivity of the intravesicular $^{22}Na^+$ retained was counted by liquid scintillation spectrometry.

As shown in Fig. 2, in the absence of imposed ΔpH, little Na^+ efflux was observed in both wild-type and SK001 strains. The imposition of ΔpH produced a great acceleration of Na^+ efflux in the wild-type, while the Na^+ efflux was more weakly accelerated in SK001. Thus it can be concluded that disruption of *yufT* decreased the Na^+/H^+ antiport activity.

Since SK001 showed a low Na^+ excretion capacity, the strain is expected to become more sensitive to Na^+ than the wild-type. The effect of the external NaCl concentration on the growth of the wild-type and SK001 was determined. Growth was monitored by optical density at 600 nm. The cells of both strains were grown in strongly buffered modified L broth (LBT medium) containing 10 g/l tryptone, 5 g/l yeast extract, 100 mM Tris–HCl (pH 7 or 8), and various concentrations of NaCl indicated. As shown in Fig. 3, at pH 7, both strains grew with an identical specific growth rate without added Na^+.

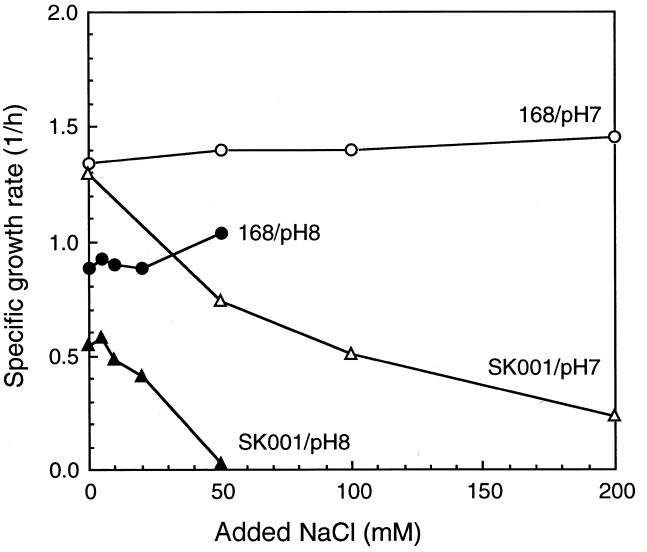

Fig. 3. Effect of NaCl on the growth of wild-type and SK001. Cells of wild-type (circles) and SK001 (*ΔyufT*, triangles) were grown in LBT medium containing various concentrations of NaCl indicated at pH 7 (open symbols) and pH 8 (closed symbols). Growth was followed at 600 nm.

The specific growth rate of SK001 was decreased with an increase in NaCl concentration up to 200 mM, while that of the wild-type was not changed. At pH 8, the specific growth rate of the wild-type was less than that at pH 7, but it was not inhibited by the addition of NaCl up to 50 mM. The growth of SK001 was significantly impaired by the elevation of pH, and more sensitive to NaCl, and no longer grew in the presence of 50 mM NaCl. No inhibitory effect on the growth of SK001 was observed when NaCl was replaced by KCl at both pH 7 and pH 8 (data not shown). These results indicated that disruption of the *yufT* gene increased sensitivity to growth inhibition by NaCl, which became more severe at elevated pH. The increased sensitivity to Na^+ with elevation of pH could be explained by an assumption that leak of Na^+ into the cell, whether carrier mediated or not, increases with increasing pH [15].

The growth rate of SK001 at pH 8 was lower than that of the wild-type in LBT medium without added NaCl. Atomic absorption analysis revealed that the LBT medium contains 12 mM of endogenous contaminating Na^+. Therefore, it was unclear that this impaired growth of SK001 is due to the effect of either pH or NaCl. In order to eliminate the Na^+ effect, we performed growth studies with minimal

salts (MM) medium containing 100 mM Tris–HCl buffer, 1 mM potassium phosphate, 1 mM $MgSO_4$, 0.1% potassium citrate, 0.2% $(NH_4)_2SO_4$, 0.05% glucose, 0.05% yeast extract, and 50 μg/ml tryptophan at pH 8. The MM medium contains 0.2 mM of endogenous Na^+. It was found that the wild-type and SK001 grew with an identical specific growth rate (1.24 h^{-1} and 1.27 h^{-1}, respectively), indicating that the inhibition of the growth rate of SK001 at pH 8 in LBT medium without added Na^+ is due to the contaminating Na^+. Since the wild-type grew fully in Na^+-eliminating MM medium at pH 8, the wild-type does not seem to require Na^+ for growth and pH homeostasis at pH 8. There is a possibility that the endogenous K^+ (10 mM in the MM medium) is used for pH homeostasis at elevated pH.

Considering these results, we conclude that *yufT* or a cluster of genes including *yufT* constitutes a Na^+/H^+ antiport system, which has a dominant role in the extrusion of Na^+. This is the second Na^+/H^+ antiport system in *B. subtilis*, as far as we know. TetA(L) of *B. subtilis* (previously referred as TetB) was first recognized as a tetracycline efflux protein and was later shown to catalyze an electrogenic Na^+/H^+ antiport [16–18]. At present, TetA(L) is considered to have dominant roles in pH homeostasis and Na^+ resistance at elevated pH [19]. Since disruption of *tetA* (L) impairs the growth at both pH 7 and 8 even in the absence of added NaCl, the impaired growth does not seem to be due to added Na^+, and the role of TetA(L) at neutral pH has not been yet defined [19]. In contrast, since the growth of SK001 was impaired by Na^+ addition at neutral pH, it can be concluded that the major role of YufT is the extrusion of Na^+ at neutral pH. Thus, the locus *yufT*, *U*, *V*, and *D* was renamed to *ntr* (*N*a^+ *tr*ansporter) *A*, *B*, *C*, and *D*, respectively.

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